An aryl-homoserine lactone quorum-sensing signal produced by a dimorphic prosthecate bacterium

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Many species of *Proteobacteria* produce acyl-homoserine lactone (AHL) compounds as quorum-sensing (QS) signals for cell density-dependent gene regulation. Most known AHL synthases, LuxI-type enzymes, produce fatty AHLs, and the fatty acid moiety is derived from an acyl-acyl carrier protein (ACP) intermediate in fatty acid biosynthesis. Recently, a class of LuxI homologs has been shown to use CoA-linked aromatic or amino acid substrates for AHL synthesis. By using an informatics approach, we found the CoA class of LuxI homologs exists primarily in α-Proteobacteria. The genome of *Prosthecomicrobium hirschii*, a dimorphic prosthecate bacterium, possesses a *luxI*-like AHL synthase gene that we predicted to encode a CoA-utilizing enzyme. We show the *P. hirschii* LuxI homolog catalyzes synthesis of phenylacetyl-homoserine lactone (PA-HSL). Our experiments show *P. hirschii* obtains phenylacetate from its environment and uses a CoA ligase to produce the phenylacetyl-CoA substrate for the LuxI homolog. By using an AHL degrading enzyme, we showed that PA-HSL controls aggregation, biofilm formation, and pigment production in *P. hirschii*. These findings advance a limited understanding of the CoA-dependent AHL synthases. We describe how to identify putative members of the class, we describe a signal synthesized by using an environmental aromatic acid, and we identify phenotypes controlled by the aryl-HSL.

bacterial communication | *Prosthecomicrobium* | α-Proteobacteria | phenylacetate | sociomicrobiology

Many bacterial species use quorum-sensing (QS) signals as a proxy for cell density. Diffusible acyl-homoserine lactones (AHLs) are common QS signals in Proteobacteria. The AHL signals accumulate during growth and at sufficient concentrations bind cognate transcription factors, QS signal receptors, which affect transcription of genes in QS regulons (1–3).

Generally, AHL production is catalyzed by signal synthases, members of the LuxI protein family. Most known AHLs have straight-chain fatty acyl side groups of 4–18 carbons, with varying degrees of oxidation at the 3-C position (1). The substrates for characterized fatty acyl AHLs are *S*-adenosylmethionine (SAM) and an acylated-acyl carrier protein (acyl-ACP) (4, 5) from the fatty acid biosynthesis pathway.

Recently, LuxI homologs have been identified that produce AHLs signals with aromatic acid and branched amino acid side chains in three species of α-Proteobacteria: RpaI from *Rhodopseudomonas palustris* catalyzes production of *p*-coumaroyl-HSL (*p*C-HSL) (6); CinI from a photosynthetic *Bradyrhizobium* catalyzes production of cinnamoyl-HSL (cinn-HSL) (7); and BjaI from *Bradyrhizobium japonicum* catalyzes production of isovaleryl-HSL (IV-HSL) (8). These three enzymes use coenzyme-A (CoA)–activated acids rather than ACP-activated acids as substrates (8, 9). Presumably, the CoA substrates are derived from the activity of cellular acyl-CoA ligases involved in organic acid catabolism. In fact, *R. palustris* requires exogenous *p*-coumarate to produce *p*C-HSL (6). Phylogenomic analyses led to the conclusion that the CoA-utilizing family of AHL synthases resulted from an evolutionary exaptation event (10).

Relatively little is known about the acyl-CoA utilizing AHL synthases and the activities they control. Based on the sequences of the three known acyl-CoA utilizing LuxI homologs and phylogenomic analyses, there are now informatics tools, which can be used to predict whether a LuxI homolog is in the ACP- or CoA-dependent family (11–13). We became interested in the α-Proteobacterium *Prosthecomicrobium hirschii* when its genome sequence was published (14). This dimorphic prosthecate bacterium has genes coding for an AHL QS system, and we predicted the *luxI* homolog codes for a member of the CoA-utilizing family. *P. hirschii* is a saprophyte that can be isolated from freshwater lakes (15) and exhibits two different cell morphologies. Some cells have multiple long prostheca, and other cells have many short prostheca. Although there is very little known about the genus *Prosthecomicrobium*, it has been reported that the short prostheca cell type increases in relative abundance in the late logarithmic phase of growth (16). This further piqued our interest because it raises the possibility that cell type is influenced by QS.

We report here that *P. hirschii* produces phenylacetyl-homoserine lactone (PA-HSL) and uses exogenous phenylacetate for this purpose. We present evidence that the gene linked to the PA-HSL synthase gene codes for the PA-HSL receptor. This QS circuit not only positively autoregulates PA-HSL synthesis, it also controls aggregation and pigment production. We find that putative CoA-utilizing AHL synthases are common in α-Proteobacteria and rare in other Proteobacteria.

Results

The *P. hirschii* LuxI Homolog Clusters with Members of the CoA-Utilizing Family of AHL Synthases. We developed a method involving the Kyoto Encyclopedia of Genes and Genomes (KEGG) orthology (KO) database and an enzyme nomenclature (EC) populated database (13) to search for likely acyl-CoA–utilizing LuxI homologs. The KO associated with acyl-CoA–utilizing LuxI homologs (K18096) was encoded almost exclusively in genomes of α-Proteobacteria. We found 974 acyl-CoA-type *luxI* homologs

Significance

Acyl-homoserine lactone (AHL) quorum sensing (QS) has received interest as a possible target for controlling microbial activities and as a model for communication involved in coordinating activities of bacteria. Recent advances show there are two related families of AHL QS signal synthesis enzymes, which differ in whether they acquire the organic acid reaction substrate from biosynthetic or primarily catabolic pathways. Most known AHL synthases utilize fatty acids activated with an acyl carrier protein from fatty acid biosynthesis. Some AHL synthases use coenzyme-A–activated organic acids. We describe a coenzyme-A–dependent AHL synthase QS system. We also set forth an experimental approach for discovering new signals that will allow further expansion of our understanding of the diversity of AHL signaling.

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(K018096) in genomes submitted to the Joint Genome Institute Integrated Microbial Genomes (JGI IMG) repository and 957 were in α-proteobacterial genomes. This represents about 30% of the 3,010 *luxI* homologs we found in sequenced α-proteobacterial genomes. The single *luxI* homolog in the recently sequenced genome of *P. hirschii* (14) was found within the family of genes encoding putative acyl-CoA–dependent AHL synthases by KO classification and in phylogenetic analyses (Fig. 1*A* and *SI Appendix*, Table S1). The *P. hirschii luxI* homolog is linked to a gene coding for a LuxR-like AHL-responsive transcription factor. We named this pair of genes *hirI* and *hirR* (Fig. 1*B*). *P. hirschii* is a dimorphic appendeged α-proteobacterial species, which exhibits two distinct cell morphologies (Fig. 1*C*). Because *P. hirschii* is a Rhizobiales as are the *Rhodopseudomonas* and *Bradyrhizobium* species, which produce aryl- or amino AHLs, we sought to identify the HirI-produced AHL.

***P. hirschii* Produces Phenylacetyl-HSL.** As a first step toward identification of the AHL produced by HirI, we grew this bacterium in the presence of L-[1-^{14}C]-methionine. To synthesize AHLs, bacteria use the common metabolic intermediate SAM derived from methionine, and when grown in the presence of L-[1-^{14}C]-methionine, the AHL ring is labeled with a ^{14}C atom (17, 18). Because AHLs are diffusible and solvent extractable, we extracted the culture supernatant fluid with ethyl acetate and fractionated the extract by C_{18}-reverse phase HPLC. Nearly all of the radiolabel was found in a single HPLC fraction (Fig. 2*A*). As expected if the radiolabeled compound was an AHL, the radiolabeled HPLC fraction was eliminated by pretreatment with purified AiiA AHL lactonase (Fig. 2*A*). None of the synthetic AHLs we tested, including *p*C-HSL, cinn-HSL, and IV-HSL, coeluted with the radiolabeled *P. hirschii* compound. This indicated that *P. hirschii* produces an AHL, which has not been described previously.

To further characterize the putative *P. hirschii* AHL, we ethyl acetate extracted material from the supernatant fluid of a 1-L *P. hirschii* culture and collected the fraction at which the radiolabel was eluted. The material in this fraction was analyzed by high-resolution liquid chromatography tandem mass spectrometry (LC-MS/MS) and scanned for the presence of an aminobutyrolactone fragment ion (M + H) of 102, common to most AHLs (6). There was one peak with the diagnostic 102-fragment ion (Fig. 2*B*), and the mass of this peak was 220.0966, which is in agreement with the molecular formula $C_{12}H_{13}NO_3$. This formula does not correspond to any known acyl-, amino, or aryl-HSL, but does correspond to PA-HSL. Chemically synthesized PA-HSL (19) coeluted with the ^{14}C product from *P. hirschii* (Fig. 2*A*), and its LC-MS/MS profile was indistinguishable from the *P. hirschii* compound (Fig. 2*C*). This is strong evidence that *P. hirschii* catalyzes the synthesis of PA-HSL (Fig. 2*D*).

For synthesis of *p*C-HSL, *R. palustris* requires *p*-coumarate in the growth medium (6). We found that *P. hirschii* produced PA-HSL without addition of phenylacetic acid to the growth medium, but *P. hirschii* requires small amounts (0.015%) of peptone and tryptone for growth and there is likely some phenylacetic acid present in these components. Thus, it is possible that, like *R. palustris*, *P. hirschii* uses an exogenous organic acid for AHL production. It is also possible that *P. hirschii* can produce a different AHL if provided the proper organic acid exogenously. To gain insights about these two possibilities, we grew *P. hirschii* in the presence of L-[1-^{14}C]-methionine plus one of 14 different aromatic acids (*Materials and Methods*) and analyzed culture fluid extracts by HPLC. Added phenylacetic acid resulted in about three times more ^{14}C-label in the HPLC fraction that coelutes with PA-HSL, than the no addition control (Fig. 2*A*). None of the other aromatic acids affected the level of ^{14}C incorporation into the PA-HSL–coeluting peak nor did growth with these aromatic acids give rise to any other peak of radioactivity. These data indicate that *P. hirschii* requires, or at least utilizes, exogenous phenylacetic acid for PA-HSL synthesis and that the *P. hirschii* AHL is in fact PA-HSL.

The *hirR* and *hirI* Gene Products Constitute a PA-HSL QS Circuit. In most LuxR-LuxI–type QS circuits, the *luxI*-like gene is positively autoregulated by its own AHL product together with the *luxR*-like gene product (2, 20). Is this the case for *hirI-hirR*? Because a genetic system for *P. hirschii* has not been established, we addressed the question by using recombinant *Pseudomonas putida* F1. The genome of this bacterium has a high GC content similar to that of *P. hirschii*, it can use phenylacetic acid as a carbon and energy source and the first step in phenylacetate catabolism

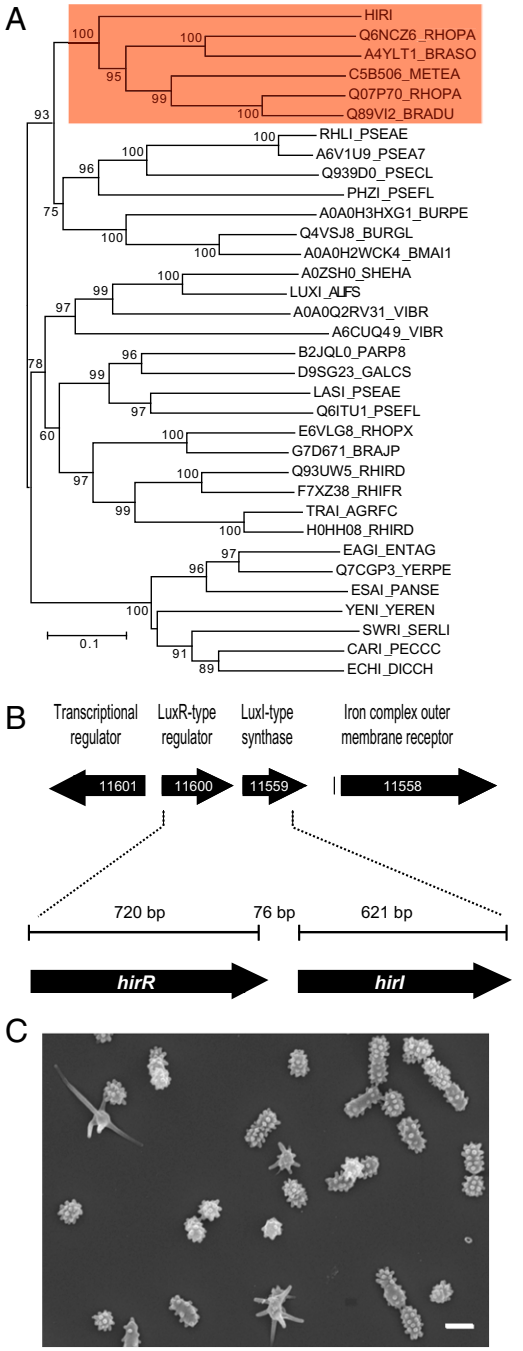

Fig. 1. The *P. hirschii* AHL synthase phylogeny, gene organization, and images of *P. hirschii* cells. (*A*) Protein phylogeny of AHL synthases. All sequences except HirI are labeled with the uniprot identifier. A description of each LuxI homolog is provided in *SI Appendix*, Table S1. The clade containing the CoA-utilizing AHL synthases is shaded in red. RpaI is Q6NCZ6_RHOPA, CinI is A4YLT1_BRASO, and BjaI is Q89VI1. The percentage that each branch was observed during bootstrap resampling (n = 1,000) is shown next to the branch. (*B*) The *hirR-hirI* genomic region. Locus designations are within the gene arrow (prefix for all is Ga0100838_ as annotated in the JGI IMG resource). (*C*) An SEM of *P. hirschii*, short-stalked cells predominate. (Scale bar: 1 μm.)

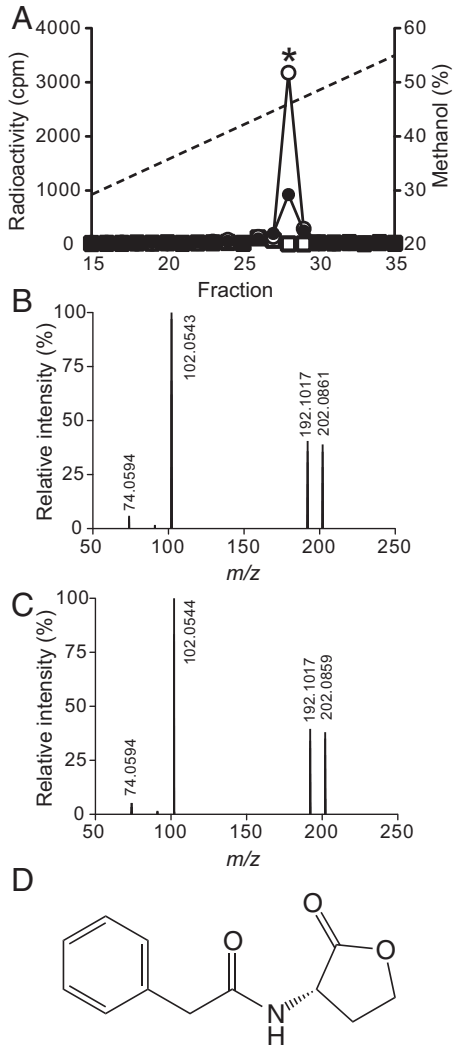

Fig. 2. Identification of the *P. hirschii* AHL. (*A*) HPLC profiles of ^{14}C-AHLs synthesized by *P. hirschii* grown in MMB with no addition (filled circles), 0.1 mM phenylacetic acid added (open circles), or AiiA lactonase added (open squares). The asterisk indicates the fraction where synthetic PA-HSL is eluted. The dashed line shows the methanol gradient. (*B*) MS/MS daughter scan of the *m/z* 220 (M + H) ion from the natural product. (*C*) MS/MS daughter scan of the *m/z* 220 (M + H) ion from chemically synthesized PA-HSL. (*D*) The proposed *P. hirschii* QS signal, PA-HSL.

involves a phenylacetyl-CoA ligase (21, 22). Finally, *P. putida* F1 does not have its own *luxI* homolog. We constructed a plasmid (pLL1) with *hirR* and a *hirI* promoter-controlled *mCherry* gene. *P. putida* (pLL1) fluorescence showed a saturable dose-dependent response to PA-HSL (Fig. 3*A*). As a control we constructed pLL2, which contained the *hirI* promoter-controlled *mCherry* but lacked *hirR*. Expression of *mCherry* was not influenced by PA-HSL in *P. putida* (pLL2) (Fig. 3*A*). This shows the response to PA-HSL is HirR dependent. Fluorescence of *P. putida* (pLL1) was unaffected by *p*C-HSL, cinn-HSL, IV-HSL, C4-HSL, C8-HSL, or 3-oxo-C12-HSL even at high (1 μM) concentrations. These findings indicate HirR responds specifically to PA-HSL with an EC_{50} of about 250 nM.

To determine whether HirI is itself a PA-HSL synthase, we ethyl acetate extracted material from *P. putida* with the *hirI* plasmid pHirI grown with or without added phenylacetate. We assayed PA-HSL concentrations in the extracted material by using *P. putida* with the *hirR-hirI* promoter-*mCherry* fusion plasmid pLL1 in a PA-HSL bioassay (*Materials and Methods*). *P. putida* (pHirI) grown in a defined medium without added phenylacetate did not produce PA-HSL, but it did when grown with added phenylacetate (Fig. 3*B*). When *P. putida* (pHirI) was grown to late-logarithimic phase in rich Luria–Bertani (LB) broth, PA-HSL levels were about 1 μM. We hypothesized that the phenylacetate acquired from the growth medium was activated by a *P. putida* phenylacetyl-CoA ligase. To test this hypothesis, we grew a strain with a *paaF* phenylacetate deletion mutation containing pHirI in LB broth as above and found no detectable PA-HSL.

PA-HSL Is a QS Signal That Controls Specific *P. hirschii* Functions in a Cell Density-Dependent Manner. As discussed above, most *luxI*-like AHL synthase genes are positively autoregulated by the AHL they produce together with the cognate LuxR homolog. Total PA-HSL levels were higher in *P. hirschii* cultures when phenylacetate was added to the medium (300 nM vs. 100 nM), but in both conditions, PA-HSL levels remained low during early and midlogarithmic growth and then increased rapidly during late logarithmic and early stationary phase (Fig. 4). This is typical of AHL systems (2, 20).

Does PA-HSL control other *P. hirschii* phenotypes? We were particularly interested in this question for several reasons. First, there is a correlation between the relative abundances of the two *P. hirschii* cell morphotypes and cell density during growth (16). Could morphological development be QS dependent? Second, we are not aware of evidence describing *p*C-HSL, cinn-HSL, or IV-HSL regulation of functions other than their own synthesis in the α-Proteobacteria, which produce these compounds. In fact, in an extensive transcriptome analysis of *p*C-HSL dependent gene transcription in *R. palustris*, we identified only a single gene activated directly by *p*C-HSL and that was the *p*C-HSL synthase gene *rpaI* itself (23). Because we were unable to manipulate *P. hirschii* genetically and delete either *hirI* or *hirR*, we approached this issue by growing *P. hirschii* in the presence of an AHL lactonase we showed degrades PA-HSL (Fig. 2*A*). Our examination of cell density-dependent cell morphology confirmed the previous report that the ratio of long to short stalked cells decreases in the late logarithmic phase of growth (16), but the PA-HSL–degrading lactonase did not influence this result (Fig. 5*A*).

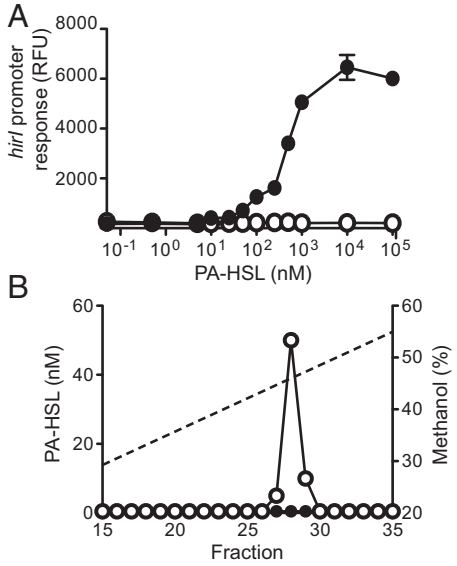

Fig. 3. HirR-HirI constitute a quorum-sensing circuit, and *P. putida* can use exogenous phenylacetate for PA-HSL synthesis. (*A*) PA-HSL–dependent *hirI* promoter activity requires HirR. Expression of *hirI::mCherry P. putida* containing pLL1 (filled circles), or pLL2 (open circles). There is a *hirR* gene in pLL1 but not in pLL2. Data are the mean, and the errors bars indicate the range of three biological replicates. (*B*) HPLC analysis of PA-HSL produced by *P. putida* (pHirI) grown in MSB broth with (open circles) or without (filled circles) 0.1 mM phenylacetate. The dashed line shows the methanol gradient.

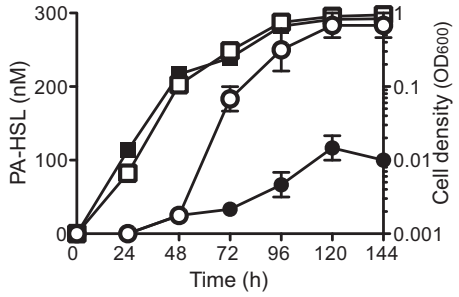

Fig. 4. PA-HSL is produced in a cell density-dependent manner. PA-HSL production (circles) and growth (squares) of *P. hirschii* grown with (open symbols) or without (filled symbols) 0.1 mM phenylacetate. Data are means, and the errors bars indicate the range of three biological replicates.

Although cell morphology appeared to be independent of PA-HSL, some phenotypic differences between cells grown with and without the PA-HSL–degrading lactonase were obvious. Notably, when cells were grown in the presence of the lactonase, we found visible cell aggregates in the late logarithmic phase. Some of these aggregates, or flocs, were of sufficient size to settle to the bottom of unshaken culture tubes. As shown by phase contrast microscopy, cells from flocculant, lactonase-grown cultures were found primarily in large clusters compared with cells grown with no addition or grown in the presence of BSA (Fig. 5*B*). Often bacterial cell aggregation is in part due production of extracellular polymeric substances (EPS), which can be complex mixtures of polysaccharides, proteins, and sometimes DNA. EPS is also important in biofilm formation. To measure *P. hirschii* EPS, we extracted cell-free supernatant fluid by using cold ethanol and measured total sugar content in the extract by using a phenol sulfuric acid protocol (*Materials and Methods*). We found that with growth in the presence of the lactonase there was about twice as much total sugar as with growth in the absence of the lactonase (Fig. 5*C*). This lactonase-dependent increase in EPS correlated with a 50% reduction in biofilm formation as measured in a microtiter dish assay (Fig. 5*D*), suggesting that when cells aggregate, they may be less likely to attach to plastic surfaces. EPS-overproducing bacterial colonies often appear rugose or rough when grown on agar plates (24); however, when agar plates contained lactonase, we did not observe rough or wrinkly colonies (Fig. 5*E*). Rather, we observed a striking but different phenotype. Without lactonase colonies were pink, but colonies on plates with lactonase-containing agar did not develop the pink pigmentation (Fig. 5*E*). Apparently, PA-HSL QS influences cellular aggregation or flocculation, biofilm formation, and pigment production.

Discussion

We describe a LuxR-LuxI–type QS system in *P. hirschii* that synthesizes and responds to PA-HSL. It is interesting that a variety of substituted derivatives of PA-HSL have been synthesized, and several are potent antagonists and agonists of other AHL-type QS systems (19, 25–27). PA-HSL has been used as a synthetic scaffold for the design of nonnative chemical probes to study LuxR-type receptor activity (28). PA-HSL itself was not very active as an agonist or antagonist of other QS systems (26). As far as we are aware, HirI is only the second AHL synthase reported to use an exogenously supplied organic acid. In this way it is similar to *R. palustris*, which requires exogenous *p*-coumarate for *p*C-HSL production (6). That phenylacetate is incorporated directly into a bacterial signal is interesting, as this plant metabolite is known to function as a growth hormone (auxin) (29). Furthermore, phenylacetate is abundant in decaying plant matter and phenylacetate degradation pathways are present in about 16% of sequenced bacterial genomes (30).

We describe the use of an AHL-degrading lactonase during culture growth to identify PA-HSL–dependent cellular activities. Our approach is similar to those reported recently to identify QS-controlled activities in other bacteria (31, 32). Using this approach, we showed PA-HSL influences *P. hirschii* pigment production, flocculation, EPS production, and biofilm formation (Fig. 5). Control of aggregation and biofilm development is a common feature of AHL QS systems (1). In many bacteria QS enhances biofilm development, but in some bacteria, it serves as a dispersal signal as it does in *P. hirschii*. Because the *P. hirschii* QS-dependent phenotypes likely depend on availability of environmental phenylacetate, the HirI-HirR-PA-HSL system must integrate information about both bacterial population density and the availability of a specific plant metabolite. This is similar to the situation with the *p*C-HSL system in *R. palustris*, but other than control of its own production, we do not know what the *R. palustris* *p*C-HSL regulates (6, 23). The *P. hirschii* system represents a model to begin to understand the biological relevance of systems, which integrate QS with other environmental factors, directly.

We hypothesize the substrate for PA-HSL synthesis is phenylacetyl-CoA for several reasons: (*i*) HirI is in the family of CoA-utilizing LuxI homologs (K18096) and groups with the CoA-utilizing enzymes (Fig. 1*A*). (*ii*) The first step in phenylacetate degradation pathways is ligation with CoA via a PA-CoA ligase enzyme (EC 6.2.1.30) (21, 22). The *P. hirschii* genome encodes three putative

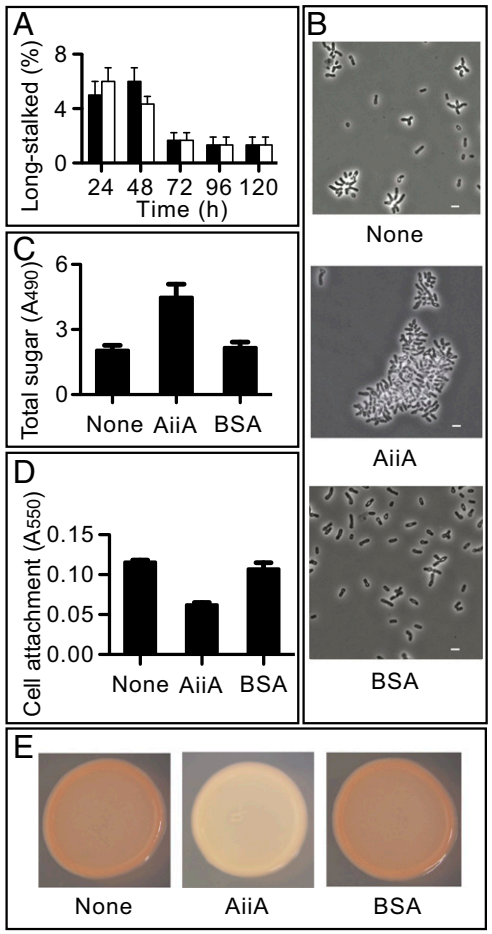

Fig. 5. PA-HSL is a QS signal that controls specific *P. hirschii* functions. (*A*) Percentage of long-stalked *P. hirschii* cells grown with (black) or without (white) AiiA lactonase over time. One hundred cells were scored for each sample. The bars show the range of three biological replicates. (*B*) Phase contrast micrographs of *P. hirschii* cells from late logarithmic phase cultures. (Scale bars: 1 μm.) (*C*) Total sugar in EPS extracted from *P. hirschii* late logarithmic phase cultures (72 h). Data are means and ranges for three biological replicates. (*D*) Biofilm biomass of *P. hirschii* (72 h). Data are the means and ranges of four biological replicates. (*E*) Pigmentation of *P. hirschii* cells grown on agar for 10 d (inoculum was a 10-μL spot of a late-logarithmic phase culture). The colonies measured ~5 mm in diameter. In *B*–*E*, None indicates no addition to the culture broth, AiiA indicates addition of lactonase, and BSA indicates addition of BSA as a control.

PA-CoA ligases, including one (Ga0100838_112492) that has 65% amino acid sequence identity with the sole PA-CoA-ligase (PaaF) in *P. putida* F1 (21). (*iii*) When *P. putida* with a *hirI* expression vector is grown with exogenous phenylacetate it produces PA-HSL, but under the same growth conditions a *P. putida* PaaF mutant does not produce PA-HSL. There remains a formal possibility that in *P. hirschii* and *P. putida* there is conversion of phenylacetyl-CoA to phenyacetyl-ACP. This conversion has been demonstrated in vitro with an enzyme from *Escherichia coli*, phenylacetyl-CoA, and *Streptomyces* ACP (33).

The ability to utilize the EC and KEGG classification systems to identify potential CoA-utilizing LuxI homologs is a valuable tool for those interested in exploring AHL signal diversity. A previous analysis of LuxI diversity using the Enzyme Function Initiative-Enzyme Similarity toolkit (34) with BjaI as a seed sequence is another useful method for predicting CoA-type LuxI homologs (9). However, such a protocol requires additional analysis, while the EC and KEGG ortholog resources are already integrated in some genome annotation pipelines (e.g., JGI IMG; https://img.jgi.doe.gov/). Our finding that CoA-type *luxI* homologs are prevalent and quite restricted to genomes in α-Proteobacteria indicates that the CoA-dependent signal synthases are common among this group of Proteobacteria. Because bacterial degradation of plant metabolites often includes a CoA-activation step (35) and α-Proteobacteria have been shown to be enriched in plant tissues (36, 37) and environments rich in plant material (38), perhaps it is logical that when the exaptation event resulting in a CoA-utilizing LuxI enzyme occurred in the lineage, this provided ample opportunity to diversify the CoA-derived AHL substrate range.

Our genomic analysis indicates the potential for discovery of novel CoA-derived AHL signals is high, but how might signal diversity be studied more systematically? One key is to utilize the radiolabel methionine protocol (*Materials and Methods* and Fig. 2*A*) for AHL screening. In principal this protocol should allow detection of AHL biosynthesis regardless of the nature of the acyl group as long as there is cellular uptake of methionine (17). The AiiA lactonase treatment provides a confirmation that a radiolabeled product is an AHL (Fig. 2*A*). We believe an additional informatics step will enhance discovery. It is clear that for some LuxI homologs exogenous acyl substrate is required or at the very least enhances AHL production, as is the case for *P. hirschii*. There are 55 categories of CoA ligases. A useful strategy may involve determining the CoA ligase inventory encoded in the genome of a bacterial species of interest, and growing cells with radiolabeled methionine and a mix of putative CoA-ligase substrates for analysis.

Here, we not only describe an AHL QS system and some functions it controls, but we describe an approach to discovery of other AHL QS systems. We imagine that there is a large amount of untapped AHL-type signal diversity among the α-Proteobacteria with virtually any organic acid for which there is a CoA-ligase serving as the acyl-group for an acyl-CoA–dependent LuxI homolog. As long as the AHL product is reasonably hydrophobic and reasonably water-soluble it can serve as a QS signal.

Materials and Methods

Acyl-HSL Synthase Phylogeny. For phylogenetic tree construction, the analysis was similar to that described elsewhere (10). Sequences were aligned with MUSCLE (39), trimmed with the alignment editor of MEGA7 (40), and the evolutionary history was inferred by using the neighbor-joining method (40) with evolutionary distances computed using the p-distance method in MEGA7.

Bacterial Strains and Growth Conditions. Strains and plasmids are described in *SI Appendix*, Table S2. With the exception of the cells prepared for scanning electron microscopy (SEM), *P. hirschii* was grown in minimal medium broth, which consisted of 0.15 g of peptone, 0.15 g of yeast extract, 1.0 g of glucose, 0.25 g of ammonium sulfate, 20 mL of Hutner's modified salts solution, 10 mL of vitamin solution per liter (41) at 26 °C with shaking. For SEM, *P. hirschii* was grown in peptone yeast extract (PYE) broth (42), which consisted of 2 g of peptone, 1 g of yeast extract, 0.3 g of $MgSO_4{\cdot}7H_2O$, 70 mg of $CaCl_2{\cdot}2H_2O$ per liter of tap water. *E. coli* was grown in LB or Terrific Broth (TB) (43). *P. putida* was grown in LB broth or mineral salts base (MSB) broth (44) plus 10 mM succinate at 30 °C with shaking. Antibiotics were used as needed at the following concentrations: 100 μg/mL ampicillin and 15 μg/mL gentamicin. For plating, media contained 1.5% agar.

Chemicals. AHLs tested for HirR activity (1 μM concentrations unless otherwise indicated): PA-HSL (45), *p*C-, and cinn-HSL (Syntech Solution LLC); IV-HSL (8), C4-, C8-, and 3-oxo-C12-HSL (Cayman Chemicals). Where indicated, the following aromatic salts (pH 7) were added to media at a final concentration of 0.1 mM: phenylacetic acid, benzoate, *p*-coumarate, *m*-coumarate, *o*-coumarate, caffeate, cinnamate, ferulate, *p*-hydroxybenzoate, methoxycinnamate, phenylalanine, sinapate, tryptophan, or vanillate.

Radiotracer Analysis of AHLs. A ^{14}C-radiotracer assay (17, 18) was used to detect AHLs produced in *P. hirschii* as follows: The inoculum for these experiments was 1 mL of a midlogarithmic phase culture (OD_{600} of 0.5) in 5 mL of fresh medium in 15-mL plastic tubes (including any aromatic acid or AiiA lactonase additions), to which 5 μCi of L-[1-^{14}C] methionine (American Radiolabeled Chemicals) was added. After 24 h at 30 °C with shaking, AHLs were extracted from total cultures with two equal volumes of acidified ethyl acetate. Ethyl acetate extracts were separated by C_{18} reverse-phase HPLC in a 10–100% methanol gradient as described previously (17), except that the flow rate was 0.75 mL per min. One-minute fractions were collected, a portion of which were mixed with 4 mL of scintillation mixture (3a70b; Research Products International), and radioactivity determined by liquid scintillation counting. To develop a protocol for further purification (see below) the radiolabel-containing fraction (fraction 28, Fig. 2*A*) was concentrated and subjected to isocratic HPLC (20% methanol-in-water). One-minute fractions were collected, radioactivity counted, and the ^{14}C-AHL made by *P. hirschii* was eluted in fractions 33 and 34.

AiiA-Lactonase Treatment. AiiA lactonase was purified as a maltose-binding protein (MalE) fusion protein from *E. coli* cells grown in TB plus 0.2% glucose by using amylose resin affinity purification as described previously (46). Purified AiiA lactonase was added every 24 h (100 μg/mL) to *P. hirschii* cultures. Control cultures included *P. hirschii* grown without added lactonase and *P. hirschii* with BSA (100 μg/mL) added every 24 h in place of lactonase. For agar plates AiiA or BSA (100 μg/mL) was added to agar cooled to 55 °C before pouring plates.

Purification and Identification of PA-HSL. We purified the *P. hirschii* AHL from 1 L of culture fluid from which cells had been removed by centrifugation. Purification was guided by results of our radiotracer experiments, and the HPLC fractionation was similar to that described elsewhere (8) except flow rate was 0.75 mL/min, we used the 30% and 50% methanol cuts from the C_{18} Sep-pak cartridge (Waters) step, and the second, isocratic HPLC was with 20% methanol. Purified material and synthetic PA-HSL were analyzed by LC-MS/MS in a Waters Acquity UPLC C_{18} RP column (1.7 μM, 2.1 mm × 30 mm) with a Thermo Linear Trap Quadrupole Orbitrap MS System (collision energy = 40 eV, cone = 35 V).

Plasmid and Strain Construction. Primers are described in *SI Appendix*, Table S3. To construct the pLL1 *hirR-*P_{hirI}*-mCherry* plasmid, we combined the following three PCR fragments by using *E. coli* DH5α-mediated assembly (47): (*i*) the *hirR* ORF plus the downstream 86-bp intergenic region containing the *hirI* promoter (PCR amplified with the hirR-F and hirR-R primers and *P. hirschii* genomic DNA as template), (*ii*) the *mCherry* ORF and ribosomal binding site (RBS) (PCR amplified with the primers mCherry-F and mCherry-R, and pUT18-mini Tn7 as template DNA), and (*iii*) PCR-linearized $pBBR_{gdh}$ (PCR amplified by using the primers pBBR-hirR-F and pBBR-mCherry-R). All primers contained 25 bp of homologous overlap, which is required for *E. coli* DH5α-mediated assembly. To create pLL2 we used the pBBR-mcherry-R and pBBR-mcherry-F as primers for PCR amplification a PCR fragment lacking the *hirR* gene from pLL1 and assembled it in *E. coli* DH5α. To create pHirI, we combined a 633-bp *hirI* DNA fragment including the *hirI* RBS) that was PCR amplified from genomic DNA by using the hirI-F and hirI-R primers with a 4,207-bp fragment of $pBBR_{gdh}$ PCR amplified by using the primers pBBR-hirI-F and pBBR-hirI-R). To construct pHirI we used *E. coli*-mediated assembly as above. Transformants were selected on LB-gentamicin agar plates and plasmid DNA sequences were confirmed by DNA sequencing. The sequence-confirmed plasmids were then used to electrotransform *P. putida*.

PA-HSL Bioassays. AHLs were extracted from broth cultures with two equal volumes of acidified ethyl acetate (0.1 mL of glacial acetic acid per liter of solvent). Levels of PA-HSL from crude extracts or HPLC fractions were measured by using *P. putida* (pLL1) as follows: Cell culture extracts, HPLC fractions, or synthetic AHLs (used to generate a standard curve) were added to 16-mm glass tubes and the solvent removed by evaporation under N_2 gas. One milliliter of *P. putida* pLL1 (subcultured from an overnight culture to an OD_{600} of 0.01 in fresh LB broth) was added to each tube and incubated for 16 h at 30 °C with shaking. To measure fluorescence, we transferred 150-μL samples to wells of 96-well black microtiter plates. mCherry fluorescence (587 nm excitation, 610 nm emission) was measured by using a Biotek H1 plate reader. Standard curves were generated by measuring the fluorescence response to different concentrations of synthetic PA-HSL.

Exopolysaccharide and Biofilm Assays. Exopolysaccharide was extracted from *P. hirschii* cell culture fluid as described elsewhere (48). Briefly, bacteria were grown to midlogarithmic phase in MMB broth with or without added AiiA lactonase or BSA (100 μg/mL each) as indicated. Two milliliters of cell-free culture fluid were extracted with three volumes of cold ethanol, and the extract was incubated at −20 °C overnight. The ethanol-precipitated EPS was collected by centrifugation (16,800 × *g* for 30 min at 4 °C), and the pellet was suspended in 100 μL of Milli-Q water. We measured total sugar by using phenol-sulfuric acid; briefly, 50 μL of suspended EPS pellet, 150 μL of concentrated sulfuric acid, and 30 μL of 5% phenol (wt/vol) were mixed in 96-well microtiter plate wells and sugar content was measured as A_{490} in a Biotek H1 plate reader. To assess the role of PA-HSL in biofilm development we used a crystal violet assay (49). Briefly logarithmic phase *P. hirschii* cells were used to inoculate MMB broth (starting OD_{600} of 0.05) with or without added AiiA lactonase or BSA (100 μg/mL). The inoculated broth was dispensed (100 μL) into wells of a 96-well polyvinyl microtiter plate. After 72 h, 30 °C wells were washed twice with Milli-Q water and then stained with crystal violet (125 μL of a 0.1% wt/vol solution) for 15 min, rinsed four times with water, and dried. Crystal violet bound to the wells was measured by dissolving it in 30% acetic acid and measuring A_{550} with a Biotek H1 plate reader).

Microscopy. For SEM imaging, *P. hirschii* was grown in PYE, cells were fixed with 3% glutaraldehyde and 1% osmium tetroxide, coated with palladium-gold (80:20), and imaged as with a JEOL-JSM 5800 electron microscope operating at 15 kV (16). Phase contrast microscopy employed an inverted Nikon Eclipse 80i Light microscope with a 100× oil immersion lens, a Digital Sight-series camera, and Nikon NIS-Elements imaging software. Photographs of *P. hirschii* cells growing on agar plates (Fig. 5*E*) were taken with a digital camera mounted on an Olympus SZX12 dissecting microscope.

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